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Morphological Changes of Cultured Endothelial Cells after Microinjection of Toxins That Act on the Cytoskeleton

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***Clostridium novyi* alpha-toxin and *C. difficile* toxins A and B (all 200 to 300 kDa) and *C. botulinum* C2-I toxin (50 kDa) caused a delayed and persistent retraction and rounding of microinjected cells. Microinjected phalloidin acted fast and reversibly. Unlike C2-I toxin, phalloidin passed through the intercellular junctions. Specific antitoxin applied to the medium did not prevent the action of microinjected *C. novyi* or *C. difficile* toxin B. Microinjected antitoxin protected against the toxins applied with the medium or injected into the same cells.**

Cytoskeletal microfilaments are the targets of three classes of toxins. Phalloidin (789 Da) forms a complex with and stabilizes F-actin (13). C2 toxin (50 kDa) from *Clostridium botulinum* is the prototype of toxins that ADP-ribosylate G-actin (1). The cytoskeletal responses to the so-called large (200- to 300-kDa) clostridial cytotoxins, i.e., *Clostridium novyi* alpha-toxin, *C. difficile* toxins A and B, and *C. sordellii* lethal toxin are not yet understood (2, 4, 5, 9). *C. novyi* alpha-toxin leads to a disintegration of the microfilaments and to a redistribution of F-actin and vinculin, but the effects of alpha-toxin are much less strong on vimentin and tubulin (9). The changes in cell shape resemble those caused by C2 toxin.

The three classes of toxins also differ in pharmacokinetics. Phalloidin passes the membranes via the bile acid transporter and is therefore targeted to hepatocytes (7). The ADP-ribosyltransferase of C2 toxin, called C2-I, requires a cofactor (C2-II) for transmembrane passage (1). The large cytotoxins act from outside without a cofactor. Their site of action, however, is probably located within the cell. Consequently, processing to an ultimate toxin on the apparently endosomal pathway into the cytosol has been suggested for *C. difficile* toxins A (for a review, see reference 5) and B (6). *C. novyi* toxin inhibits the meiotic maturation of *Xenopus* oocytes when applied by injection but not when applied extracellularly (3). This experiment has shown for the first time that a nonendocytosed toxin acts within the cell; however the system is too complex to use for elucidating the mode of action.

We describe herein the microinjection of the toxins into endothelial cells. With this technique it is possible to determine whether the route of application influences the morphological responses and to study protection to the toxin by cross-application of antitoxin through the alternative route. Passage through gap junctions (8) can be detected by observing cytopathic responses in neighboring noninjected cells.

Cells were cultured from porcine pulmonary artery (9, 10) in 35-mm dishes. They were grown to confluency in medium M199 with 15% fetal calf serum and were used between the first and fifth passages. To facilitate localization of the injected cells, grids were engraved into the surfaces of lettered dishes. The marking comb consisted of 15 steel needles, approximately 100 μm apart. After seeding, the cells grew over the scratches and connected adjacent fields by gap junctions. The microinjection system consisted of an Eppendorf (Hamburg, Germany) micromanipulator 5170, a microinjector 5242, and capillaries with an inner tip diameter of about 0.5 μm. The holding pressure was 30 hPa, the injection pressure was 80 hPa, and the duration of injection was approximately 250 ms. The injection volume (approximately 10 fl) was about 1% of the cell volume. Since the final intracellular concentration cannot be accurately calculated, the initial concentration of the substances injected will be given. Mechanical destruction by the injection procedure was rare (about 1% of the cells) and always rapid. It could never be mistaken for the slow, distinct toxin effects. The toxins used were described in detail previously (2, 4, 11). The polyclonal antiserum against *C. novyi* toxin was from mice (2, 4), and the antiserum against *C. difficile* toxin B from goats. The substances to be microinjected were dissolved in phosphate-buffered saline with 0.1% bovine serum albumin as a protective colloid. Potassium glutamate (140 mM) buffered with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (10 mM, pH 7.2) led to the same results.

The three large clostridial cytotoxins acted after a latency period, which was concentration dependent and was never shorter than about 20 min, even at the highest concentrations applied (Fig. 1). The kinetics and the morphological changes after microinjection were the same as those achieved after extracellular application. Retraction of injected cells was the first visible event, followed by a rounding up of the cell, with many cytoplasmic extensions still adhering to the supports (Fig. 2C, D, and E). The cells could no longer recover at this stage; they were first displaced and then replaced by dividing cells from noninjected fields. Applied extracellularly, *C. novyi* toxin was about equipotent with *C. difficile* toxin A and about 100 times less potent than *C. difficile* toxin B, as shown by inhibition of uridine incorporation (4). Similar relative potencies were found, based on changes in cell shape, after injection of graded (1:10) doses of the three toxins. The detection limits for morphological changes were 400 ng/ml for *C. novyi* toxin and *C. difficile* toxin A, 40 ng/ml for *C. difficile* toxin B, and between 4 and 0.4 ng/ml for C2-I toxin. Some cells were stained for F-actin with fluorescent phalloidin (9) about 3 h after injection of *C. novyi* toxin (4

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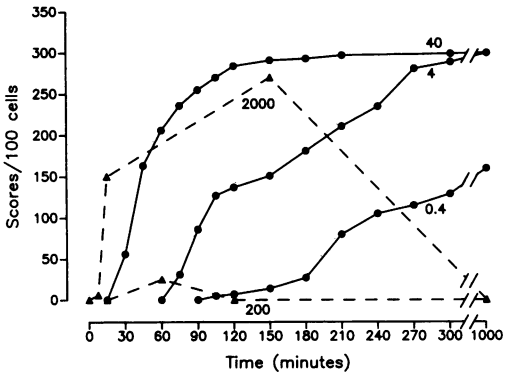

FIG. 1. Time-dependent morphological changes caused by injected *C. novyi* toxin (●) or phalloidin (▲). About 100 cells grown to confluence were injected with the toxins (concentrations in micrograms per milliliter are indicated) and scored visually (ordinate) at different times after injection (abscissa). Cells injected with *C. novyi* toxin were given one point when slightly retracted, two points when strongly retracted, and three points when rounded. Cells injected with the higher phalloidin concentration became polymorphous within the first hour and round thereafter. Note the complete recovery overnight from phalloidin poisoning, whereas the changes caused *C. novyi* toxin continued.

μg/ml). The changes were the same as those reported previously (4) for cells that had been toxified extracellularly. The action of injected *C. novyi* or *C. difficile* toxin B could be prevented by preinjection of the cells with the respective immune serum (data not shown).

To assess reversibility of the toxin-induced alterations, microinjection of *C. novyi* or *C. difficile* toxin B was followed by injection of the respective antitoxin into the same cells after different time periods. Antitoxin injected within the latency period of about 30 min prevented the expected morphological alterations. When applied thereafter, it reverted the initial toxin effect within hours. It was not possible to assess reversibility with cells in later stages because their membranes had become too rigid for additional injection. Microinjection permitted alternate applications of toxin and antitoxin. When present in excess within the cell medium, *C. novyi* antiserum (1:100, vol/vol) did not diminish the action of subsequently microinjected toxin (4 μg/ml). Conversely, microinjected antiserum, but not normal mouse serum at the same concentration, prevented the action of extracellularly applied *C. novyi* toxin (Fig. 3). Alternate application of *C. difficile* toxin B and its antiserum led to the same outcome, indicating that the target(s) of both toxins is located within the distribution volume of the microinjected antitoxins.

The smallest amount of microinjected C2-I toxin that resulted in visible morphological changes was about 1 ng/ml. The latency period was shorter, whereas the time course and morphological changes (Fig. 2B) were the same as those for the large clostridial cytotoxins. There was no morphological evidence for transcellular spread of toxin from injected single cells. As expected, C2-I toxin (4 μg/ml) and phalloidin (2 mg/ml) were inactive extracellularly.

Microinjected phalloidin at about 0.2 mg/ml resulted in visible changes (Fig. 1). They occurred within minutes, reached a maximum within about 2 h, and then waned; the cells recovered completely within about 24 h. The cell shape became polymorphous after initial or modest poisoning, quite unlike the changes resulting from the bacterial toxins.

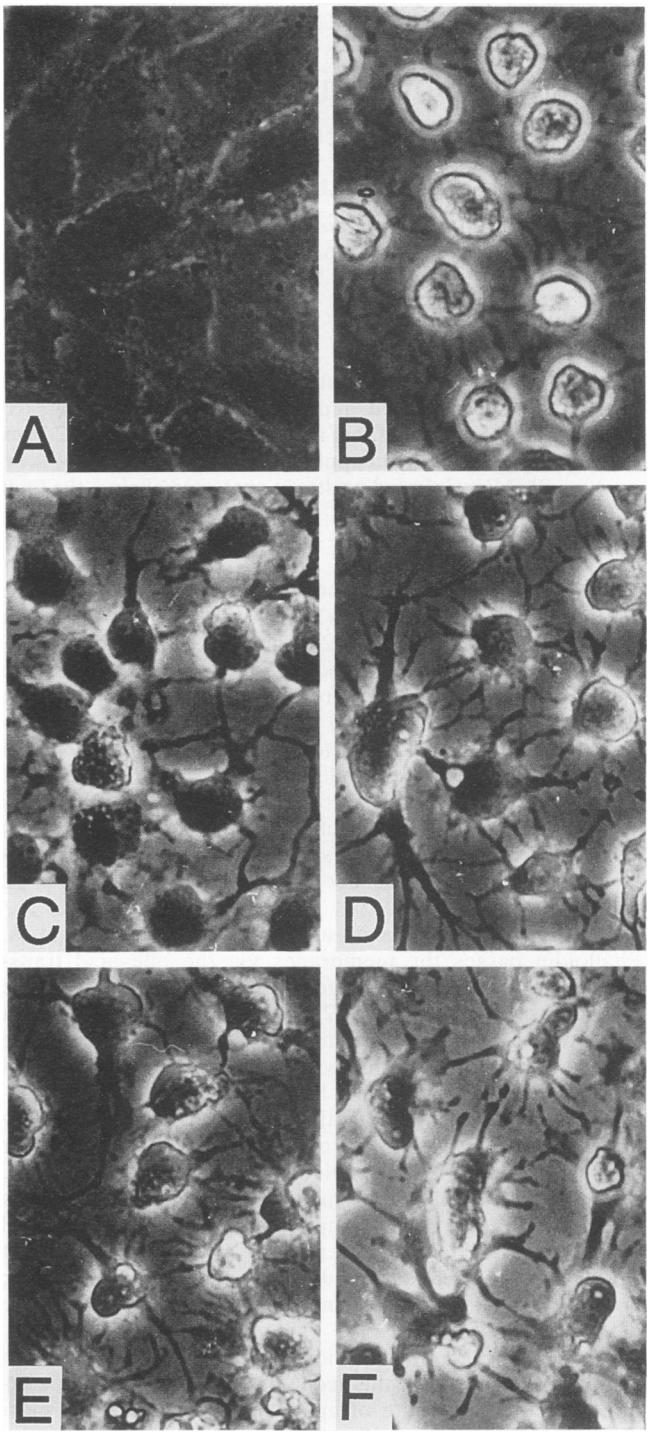

FIG. 2. Endothelial cells after injection of high concentrations of clostridial cytotoxins and phalloidin. (A) Without toxin; (B) *C. botulinum* C2-I toxin (400 ng/ml, 5 h); (C) *C. difficile* toxin B (400 ng/ml, 24 h); (D) *C. difficile* toxin A (40 μg/ml, 24 h); (E) *C. novyi* toxin (40 μg/ml, 24 h); (F) phalloidin (2 mg/ml, 5 h).

High phalloidin concentrations (2 mg/ml) caused rounding and retraction (Fig. 2F) as described above for C2-I toxin and the large clostridial cytotoxins. Membrane blebbing was more pronounced. Cells close to those injected with phalloi-

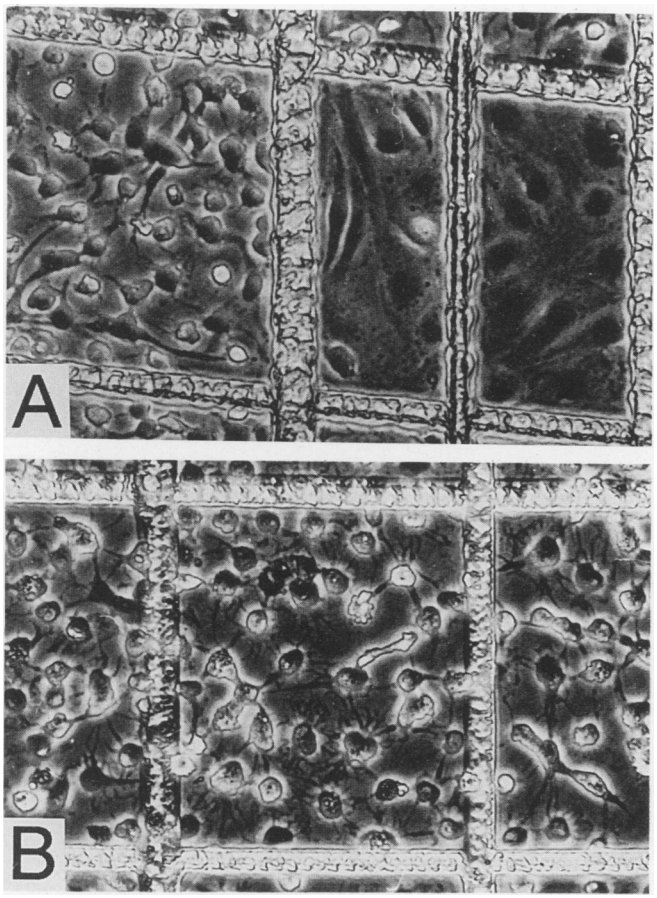

FIG. 3. Prevention of the action of extracellularly applied *C. novyi* toxin by previous antitoxin injection. (A) The cells in the two rectangles on the right were injected with mouse antiserum (1:10) before the culture was exposed to externally applied *C. novyi* toxin (1 μg/ml) for 2 h. (B) Same experiment as in panel A, except that normal mouse serum (1:10) was injected into the cells within the central rectangle. Essentially the same picture emerged when the cells were injected with goat antiserum against *C. difficile* toxin B (1:10) and then the toxin (200 ng/ml) was applied externally. Conversely, neutralizing concentrations of *C. difficile* antitoxin in the bath solution did not protect against *C. difficile* toxin B (400 ng/ml) injected into the cell. Both antisera were tested in mice and in cell cultures. Concentrations at least 100-fold greater than neutralizing concentrations were employed.

din also became polymorphous. Since the peptide was inactive when applied to the medium, it appeared to spread through gap junctions. Accordingly, Lucifer yellow (30 mg/ml; Sigma, St. Louis, Mo.) stained the cells up to 10 cell diameters distant from the injection site in a confluent layer. Gap junctions in cultured endothelial cells are known to allow transfer of electrical impulses, dyes, and nucleotides (8). It can now be added that they serve as intercellular channels, transferring small toxins over distances spanning 5 to 10 cells.

The cells did not respond to two-chain tetanus toxin, its heavy or light chains, or to botulinum A neurotoxin (all injected at 100 μg/ml). Cell death caused by diphtheria toxin, however it was applied, was preceded by rounding and detachment. Retraction was absent.

The responses to injection of the three large clostridial cytotoxins and to C2-I toxin are indistinguishable with respect to morphology and progress with time; together with the common structural manifestations at the microfilament system (9, 12), this suggests a functionally related target. Certainly, the biochemical modes of action are not identical, because the large toxins lack ADP-ribosyltransferase activity. It remains to be determined whether the destruction of the microfilament system is caused by a direct interaction with the large clostridial cytotoxins or by an indirect event taking place elsewhere, like a tent collapsing when its lines are severed. An indirect effect might be elicited by *C. botulinum* C3 toxin, which ADP-ribosylates *ras* proteins and nevertheless alters the microfilaments (12).

The cellular pharmacokinetics of the toxins can also be traced by microinjection. The target of *C. novyi* and *C. difficile* toxin B has been located within cytoplasmic compartments. Microinjection also shows that neither the large clostridial cytotoxins nor C2 toxin needs to be processed during endocytosis or pinocytosis. These pathways are circumvented by microinjection into endothelial cells but are relevant in extracellular poisoning (5, 6). C2-I toxin is retained in the injected cell, where it may ADP-ribosylate any G-actin that is freshly synthesized. Both retention and action may contribute to the persistence of the morphological changes. The rapid and reversible response to phalloidin might be terminated by diffusion of free peptide through gap junctions into the endothelial syncytium. Moreover, F-actin might be assembled de novo in excess over free phalloidin.

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REFERENCES

1. **Aktories, K., and A. Wegner.** 1989. ADP-ribosylation of actin by clostridial toxins. J. Cell Biol. **109:**1385–1387.
2. **Bette, P., J. Frevert, F. Mauler, N. Suttorp, and E. Habermann.** 1989. Pharmacological and biochemical studies of cytotoxicity of *Clostridium novyi* type A alpha toxin. Infect. Immun. **57:** 2507–2513.
3. **Bette, P., F. Mauler, C. Mohr, and E. Habermann.** 1990. Microinjection of α-toxin from *Clostridium novyi* type A promotes meiotic maturation in *Xenopus laevis* oocytes. Toxicon **28:**1368–1371.
4. **Bette, P., A. Oksche, F. Mauler, C. von Eichel-Streiber, M. R. Popoff, and E. Habermann.** 1991. A comparative biochemical, pharmacological and immunological study of *Clostridium novyi* α-toxin, *C. difficile* toxin B and *C. sordellii* lethal toxin. Toxicon **29:**877–887.
5. **Fiorentini, C., and M. Thelestam.** 1991. *Clostridium difficile* toxin A and its effects on cells. Toxicon **29:**543–567.
6. **Florin, I., and M. Thelestam.** 1986. Lysosomal involvement in cellular intoxication with *Clostridium difficile* toxin B. Microb. Pathog. **1:**373–385.
7. **Frimmer, M., E. Petzinger, U. Rufeger, and L. B. Veil.** 1977. The role of bile acids in phalloidin poisoning. Naunyn-Schmiedeberg's Arch. Pharmacol. **301:**145–147.
8. **Larson, D. M., and J. D. Sheridan.** 1982. Intracellular junctions and transfer of small molecules in primary vascular endothelial cultures. J. Cell Biol. **92:**183–191.
9. **Oksche, A., R. Nakov, and E. Habermann.** 1992. Morphological and biochemical study of cytoskeletal changes in cultured cells after extracellular application of *Clostridium novyi* alpha-toxin. Infect. Immun. **60:**3002–3006.
10. **Suttorp, N., W. Seeger, J. Uhl, F. Lutz, and L. Róka.** 1985.

Pseudomonas aeruginosa cytotoxin stimulates prostacyclin production in cultured pulmonary artery endothelial cells: membrane attack and calcium influx. J. Cell Physiol. **123**:64–72.

11. **von Eichel-Streiber, C., U. Harperath, D. Bonse, and U. Hadding.** 1987. Purification of two high molecular weight toxins of *Clostridium difficile* which are antigenically related. Microb. Pathog. **2**:307–318.
12. **Wiegers, W., I. Just, H. Müller, A. Hellwig, P. Traub, and K. Aktories.** 1991. Alteration of the cytoskeleton of mammalian cells cultured in vitro by *Clostridium botulinum* C2 toxin and C3 ADP-ribosyltransferase. Eur. J. Cell Biol. **54**:237–245.
13. **Wieland, T., and V. M. Govindan.** 1974. Phallotoxins bind to actins. FEBS Lett. **46**:351–353.